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Research Article

A STUDY ON THE ASSESSMENT OF SAFETY OF A POLYHERBAL FORMULATION IN ACUTE DIARRHOEA

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ABSTRACT

Objective: To explore the safety profile of the proposed polyherbal formulation LQ 14 which is composed of various antimicrobial ingredients in rodents according to OECD (423) for acute oral toxicity study and OECD (407) for 28-days repeated dose toxicity study. **Materials and methods:** Acute toxicity studies and 28-days repeated dose toxicity were done on male Swiss slbino mice and Wistar albino rats. The animals were randomly divided into four groups of five animals each (n=20). In acute toxicity study, three doses of the extract (2.5-10 ml/kg body weights) were administered to 3 groups of the animals respectively. All animals were observed for 24 hours and general symptoms of toxicity and mortality were recorded. In 28-days repeated dose toxicity study, wistar male rats were grouped by randomized design and assigned to three groups of 6 animals each (n=18). Two doses of the extract (2.5, 5 ml/kg body weight) were administered daily to 2 groups. The last group was control in both and received 10, 5 ml/kg body weight of deionized water. Route of administration was oral in all the groups of animals. **Results:** No treatment related toxicity was found in both acute and repeated dose toxicity studies. **Conclusion:** The absence of biologically significant differences in the toxicological endpoints supports the safety of LQ14 in the treatment of diarrhea.

KEYWORDS: Diarrhoea, Polyherbal drug, Acute toxicity study, OECD guidelines, Toxicology, Endpoints.

INTRODUCTION

Assurance of safety, quality and efficacy of medicinal plants and herbal products is a key issue, which needs to be addressed. Both the general consumer and health care professional need up-to-date authoritative information on the safety and efficacy of phyto-medicines[1,2]. Further, conducting toxicity studies for herbal preparations is essential step that will strengthen the acceptance of herbal medicines by scientific community. The test drug, formulation LQ14 contained herbs and its parts of *Holarrhena antidysenterica, Mangifera indica, Cyperus rotundus, Centella asiatica, Curcuma longa* etc. and possess significant anti-viral, anti-protozoal, bactericidal and anti-diarrhoeal properties. All the components in the test formulation LQ 14 have been reported in Indian Ayurvedic Pharmacopia (*vide* reference) and mentioned their use as medicine before 1000 BC to present time. But as all these components are mixed in a new formulation by modern techniques (solvent extraction) hence, it is necessary to find out whether any adverse effect originated from this process of preparation of in combination. In that regards, it was considered to examine the toxicological investigations on LQ14.

MATERIALS AND METHODS

Test Formulation LQ14

The proprietary test formulation LQ14 was formulated, prepared and supplied in the liquid form in amber coloured bottle by the sponsor of the present research project. The anticipated therapeutic action is anti-diarhhoeal. The formulation contains five herbal parts and other constituents, like thickener, preservative, stabilizer, solvent etc.

The shelf-life of LQ14 is 36 months if it is preserved at 25±2ºC in dark cool bottle. The density of LQ14 is 1300 Kg/m^3 and specific gravity is 1.3 when packed. The LQ14 was analyzed for heavy metals contamination and atomic absorption spectroscopic (AAS) reports informed that LQ14 contains 2.34 ppm lead, less than 0.05 ppm arsenic, less than 0.002 ppm cadmium and 0.248 ppm mercury.

Phytoconstituents information of the test formulation [3-9]

Sl. No.	Name of Ingredients	Parts used	Activities	Amount (%)
1.	***Kurchi* (*Holarrhena antidysenterica*)**	Stem bark	Antimicrobial	100 mg
2.	***Mango* (*Mangifera indica*)**	Leaves	Antimicrobial	100 mg
3.	***Musta* (*Cyperus rotundus*)**	Rhizome	Antimicrobial	20 mg
4.	***Mandukaparni* (*Centella asiatica*)**	Whole plant	Antimicrobial	20 mg

5.	***Haridra* (*Curcuma longa*)**	Rhizome	Antimicrobial	10 mg
6.	**Pectin**	-	Thickener	4 mg
7.	**Sodium benzoate**	-	Preservative	6 mg
8.	**Citric acid**	-	Stabilizer	3 mg
9.	**Purified water**	-	Solvent	q.s.

Test Drug Preparation

The supplied test drug, LQ14 was ready to use. No other solvent was mixed throughout the study.

Reagents and Chemicals

The test drug was supplied by the sponsor (M/s Parker Robinson Pvt. Ltd., Kolkata). Castor oil (M/s Dabur India Limited, India), Charcoal (M/s Merck, India), Loperamide (Lopamide, M/s Torrent, India), Cell Diluting Fluids (M/s StanBio, India), Biological Commercial Kits (M/s Span Diagnostics Ltd, India) and other general reagents were used.

Etihcs Clearance

The study began after obtaining ethics clearance from the Institutional Ethics Committee (IEC) no: PR-HC/06 ETHICs/ 1019. The local body of CPCSEA and the IAEC were non-functioning, so IEC has provided the approval for this study. Some of the members of both IEC and IAEC were in common, hence approval was solely provided by the IEC.

Animals and animal care

In accordance with the CPCSEA guidelines, animals were allowed to be acclimatized for a period of 2 weeks in our laboratory environment prior to the study. Animals were housed in polypropylene cages (4 animals per cage), maintained under standard laboratory conditions (i.e. 12:12 hour light and dark sequence; at an ambient temperature of 25±2ºC; 35-60% humidity); the animals were fed with nutritionally balanced pellet diet for rodent and water *ad libitum*.

Acute Toxicity Studies

LQ14 was tested for acute oral toxicity study following the guidelines of OECD (423) [3,4,5]. Swiss (IB) male albino mice (25 g) and Wistar/NIN(IB) albino rats (150 g) were randomly divided into three groups of five animals each. The age of rats and mice being 12 weeks. Male animals were chosen for safety evaluation as female animals possess fluctuations in their hormonal mileu resulting from estrus cycle. Female animals were not used as most of them were pregnant. The animals were fed on mice and rats pellets and water *ad libitum*. The animals were starved for 12 hours prior to testing. Three doses of the extract (2.5-10 ml/kg body weights) were administered by oral intubation to 3 groups of the animals respectively by step-up method. Actual dose administered are mentioned in table 1.

The volume of the extract administered to each animal in the test group was calculated based on the body weight. All animals were observed for 24 h (extended upto 3 days) and general symptoms of toxicity and mortality were recorded.

28-Days Repeated Toxicity Studies [6,7]

OECD guidelines for the testing of chemicals (synthetic or natural) are periodically reviewed in the light of scientific progress, changing assessment practices and animal welfare considerations.[10,11,12] The original guideline 407 repeated dose 28-days oral toxicity studies in rodents have been adopted that is generally used for testing of herbal medicines.[13] Healthy Wistar stain male rats have been acclimatized to laboratory conditions for at least 7 days and have not been subjected to previous experimental procedures, were used. They were grouped by randomized design and assigned to two groups of 6 animals each as follows:

Group I: Control: given 5 ml/kg deionized water
Group II: Low dose: given 2.5 ml/kg LQ14
Group III: High dose: given 5 ml/kg LQ14

The polyherbal test formulation was given by oral route, once per day continuously for 28 days. The animals were maintained properly throughout the treatment. After completion of the study, the hematological analysis was conducted from living animals and thereafter, all animals were sacrificed under deep anesthesia. Blood samples were collected and preserved for biochemical estimations. The organs were excised for organ weight and histological studies. Throughout the study period, the following observations and investigations were conducted.

Food & Water Consumption: Monitoring by measuring the water in the containers was done at day 0 and on day 28. Quantity of food consumed was also estimated by given a weighed quantity of pellet diet followed by weighing the amount left over diet in the cage at the end of the 24 hours.

Body Weight: The body weights of the animals were recorded at the time allocation of animals to the different groups, and the end of the study using electronic digital weighing machine for animals' weight (5g) in fasting conditions. The weight gaining was calculated by extracting the results of initial weight and final weight.

Hematological Studies: The hemoglobin concentration was studied following to cyanomethaemoglobin method. The counts of red blood cells and white blood cells were also done using hemocytometer.

Biochemical Studies: Blood samples were collected from rats and serum was separated by centrifuge. The following parameters ware analyzed spectrophotometrically using commercial kits.

- Serum Cholesterol
- Serum Alanine transaminase (ALT)
- Serum Aspartate transaminase (AST)
- Serum Alkaline Phosphatase (ALP)
- Blood Urea Nitrogen (BUN)
- Serum Creatinine

Organ Weight: The animals were sacrificed and the vital organs i.e. heart, liver, kidney and spleen were excised, cleaned, dried and weighed in high precision electronic weighing machine. The mean values of absolute organs weight was represented as g wt. Finally, the relative organ weight ratio was determined.

Histopathological Studies

Histopathological studies on liver, kidney and spleen were conducted to find out any toxicity on these

vital drug regulatory organs. Photomicrographs of representative tissues were taken at various magnifications.

Statistical Analysis

Mean and standard error values were determined for all the parameters and the results were expressed as Mean ± SEM. The data were analyzed employing paired t-test and comparison between the groups using statistical software SPSS version 17 (SPSS Sciences, Chicago, USA).

RESULTS

In the single oral dose toxicity study of LQ14, no treatment related toxic changes were observed at the highest dose (10 ml/kg). Hence, the dose levels *i.e.,* $^1/_{4^{th}}$ (2.5 ml/kg) and $^1/_2$ (5 ml/kg) with a common ratio of 2 of the highest dose tested (10 ml/kg), were selected for 28 days repeated dose study. All experiments were conducted following the guidance of Good Laboratory Practice (GLP) for experimental animals. The observations from this study are as follows:

1. **General Observation:** All animals were examined twice daily for general appearance, behavior, and signs of toxicity, morbidity, and mortality. There was no mortality in the 28 days repeated dose oral toxicity testing (Table 1). All the animals of treated and control groups remained active and healthy during the period of the study.
2. **Food & Water Consumption:** Monitoring of food and water consumption revealed irregular variations in the average 24 hour food and water intake of animals in the control and treated groups (Table 2). However, there was no variations with the test substance, LQ14.
3. **Body Weight:** Animals in all groups gained weight and it remained fairly constant throughout the experimental period (Table 3, Fig. 1). However, no significant differences were observed among LQ14 treatment groups as compared to the control group.
4. **Hematological Studies:** The results of examination of hematological parameters of LQ14 treated and control group are shown in Table 4. No biologically significant differences in hematological profiles were observed. The hematological parameters were within normal limits.
5. **Biochemical Studies:** Examination of the serum biochemistry values of the test compound, LQ14 treated and control group is shown in Table 5. Biochemical parameters of liver function and renal function did not reveal hepatic or renal impairment following the administration of LQ14.
6. **Organ Weight:** There were no marked differences in the weights of various vital organs (heart, liver, kidney, spleen) of the rats treated with LQ14 when compared to control (Table 6). Gross examination and even microscopic study of the vital organs on autopsy did not reveal any abnormalities.
7. **Histopathological Studies:** In all groups of animals, histological architecture of the liver was found to be within normal limits. (Fig. 2-10). Hepatocytes examination did not particularly reveal any necrosis or steatosis. Portal areas were without any particularity and Kupffer's cells were not modified. It has not also been observed any fibrosis. In most observed cases, it has been observed that the normal histological structure of the spleen was respected. Further, the histological structure of the glomerulus was respected in all the observed groups of kidneys and it has neither been observed membrane thinning nor hyperplasia of mesengial cells or deposits of immunoglobulins. These observations clearly indicated that the polyherbal test formulation no toxic or side effect on tissue morphology.

Table 1: Acute oral toxicity of LQ14 in rodents

Species/ Group	Dose ml/kg	Log dose	Dead/Total N			Dead%	Corrected %	Probit
			Day 1	Day 2	Day 3			
Mice								
1	2.5	0.3979	0/6	0/6	0/6	0	4.16	3.267
2	5	0.6989	0/6	0/6	0/6	0	4.16	3.267
3	10	1.0000	0/6	0/6	0/6	0	4.16	3.267
Rats								
4	2.5	0.3979	0/6	0/6	0/6	0	4.16	3.267
5	5	0.6989	0/6	0/6	0/6	0	4.16	3.267
6	10	1.0000	0/6	0/6	0/6	0	4.16	3.267

Corrected formula for 0%= 100(0.25/n), when n was the number of animals in the group

Table 2: Effect of LQ14 on food and water consumption in rats

Group	Control		LQ14 treatment			
Dose	**5 ml/kg**		**2.5 ml/kg**		**5 ml/kg**	
Day	**0 Day**	**28 Day**	**0 Day**	**28 Day**	**0 Day**	**28 Day**
Food consumption (g/day)	20.5±0.67	36.1±0.89	22.5±0.87	35.5±2.01	19.5±0.78	35±0.49
Water consumption (ml/day)	14.5±0.80	20±0.44	12.5±0.92	19±0.62	14±0.72	20.5±1.56

N=6 rat in each group; Treatment= once/day/p.o. for 28 days; Values are Mean±SEM; Data are compared statistically by Paired test using software spss version 17 with respective group of control and showed not significant (NS)

Figure 1: Effect of LQ14 on body weight in rats

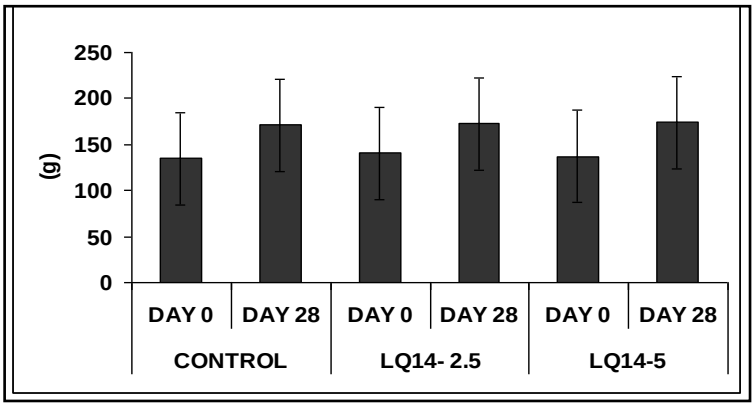

Table 3: Effect of LQ14 on body weight in rats

Group	Control		LQ14 treatment			
Dose	5 ml/kg		2.5 ml/kg		5 ml/kg	
Day	0 Day	28 Day	0 Day	28 Day	0 Day	28 Day
Body weight (g)	135±2.82	171.3±2.29	140.8±5.38	172.6±2.30	136.8±2.47	173.9±2.62

N=6 rat in each group; Treatment= once/day/p.o. for 28 days; Values are Mean±SEM; Data are compared statistically by Paired test using software SPSS version 17 with respective group of control and showed not significant (NS).

Table 4: Hematological values of rats treated with LQ14 for 28 days

Group	Control	LQ14 treatment	
Dose	5 ml/kg	2.5 ml/kg	5 ml/kg
Hb (g%)	14.51±0.14	14.59±0.19	14.57±0.11
RBC (10^6/mm^3)	7.90±0.032	7.88±0.028	7.83±0.035
WBC (10^3/mm^3)	6.77±0.039	6.81±0.047	6.82±0.057
Neutrophil (%)	45±1.38	43.8±1.16	44.5±1.11
Eosinophil (%)	3.51±0.48	3.38±0.52	3.28±0.57
Lymphocyte (%)	51±2.59	52±1.43	49.8±1.82
Monocyte (%)	2.50±0.22	2.35±0.38	2.51±0.26

N=6 rat in each group; Treatment= once/day/p.o. for 28 days; Values are Mean±SEM; Data are compared statistically by Paired test using software SPSS version 17 with respective group of control and showed not significant (NS).

Table 5: Serum values of rats treated with LQ14 for 28 days

Group	Control	LQ14 treatment	
Dose	5 ml/kg	2.5 ml/kg	5 ml/kg
Cholesterol (mg/dl)	82.1±2.92	79.3±3.98	77.8±4.58
ALT (IU/L)	34.6±0.95	31.8±1.83	34.7±1.45
AST (IU/L)	36.7±1.45	33.2±1.72	34.9±1.40
Alkaline Phosphatase (KA)	58.85±3.17	55.70±1.98	51.37±3.08
BUN (mg/dl)	8.71±0.42	8.32±0.74	8.63±0.39
Creatinine (mg/dl)	0.63±0.03	0.57±0.02	0.58±0.04

N=6 rat in each group; Treatment= once/day/p.o. for 28 days; Values are Mean±SEM; Data are compared statistically by Paired test using software SPSS version 17 with respective group of control and showed not significant (NS)

Table 6: Absolute organ weight of rats treated with LQ14

Group	Control	LQ14 treatment	
Dose	5 ml/kg	2.5 ml/kg	5 ml/kg
Heart (g)	0.459±0.008	0.455±0.007	0.458±0.006
Liver (g)	5.29±0.118	5.37±0.109	5.45±0.127
Kidneys (g)	0.558±0.006	0.551±0.007	0.549±0.008
Spleen (g)	0.452±0.005	0.446±0.004	0.446±0.008

N=6 rat in each group; Treatment= once/day/p.o. for 28 days; Values are Mean±SEM; Data are compared statistically by Paired test using software SPSS version 17 with respective group of control and showed not significant (NS)

Histopatological Findings of LQ14 treatment in the test group of rodents

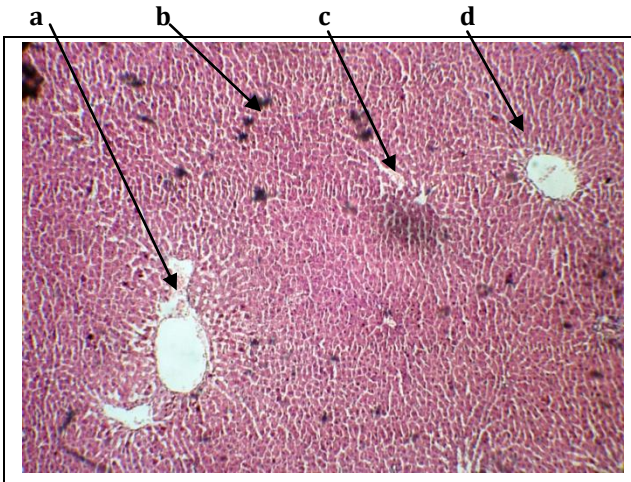

Fig. 2: Normal Liver
HE Stain
a. Central Vein
b. RBCs in sinusoids
c. Sinusoids
d. Cords of hepatocytes

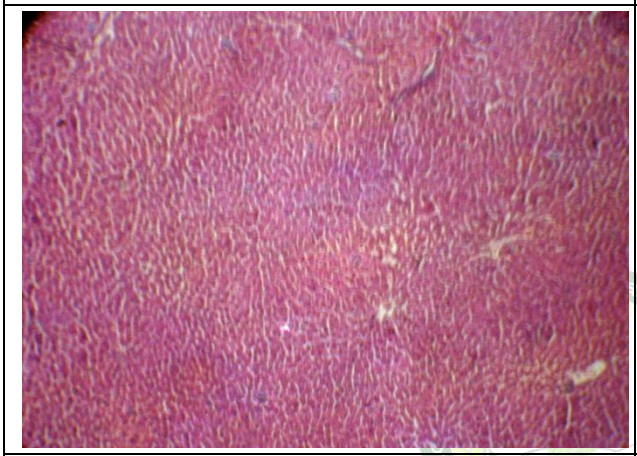

Fig. 3: LQ14 - 2.5 ml/kg induced Liver

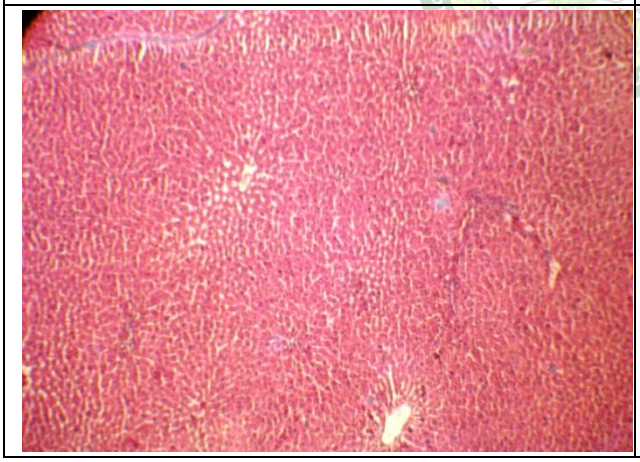

Fig. 4: LQ14 - 5 ml/kg induced Liver

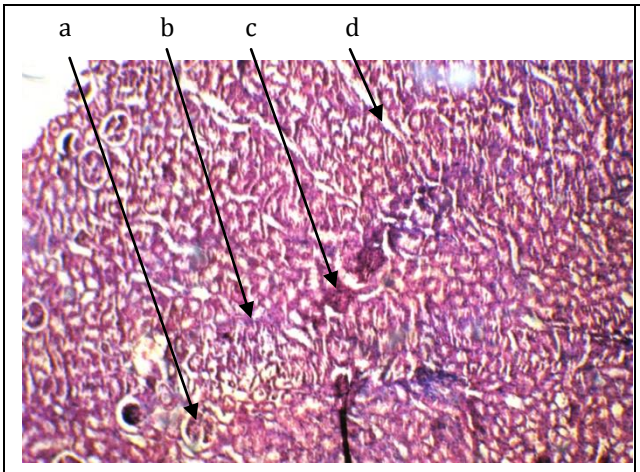

Fig 5: Normal Kidney
HE Stain

a. **Glomuruli**
b. **Blood vessels**
c. **Lumen of kidney tubule**
d. **Proximal and distal convulated tubule**

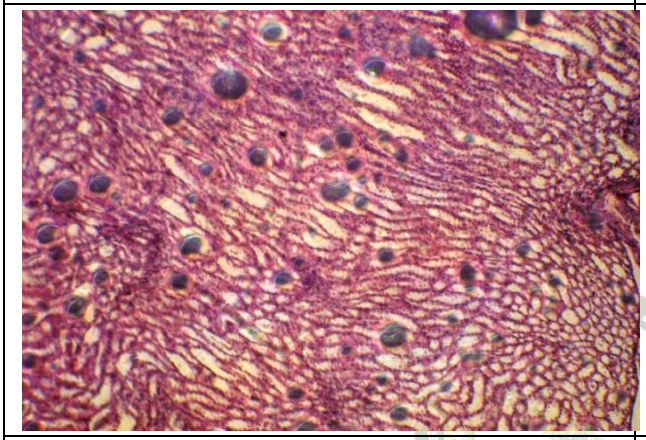

Fig. 7: LQ14 – 2.5 ml/kg induced Kidney

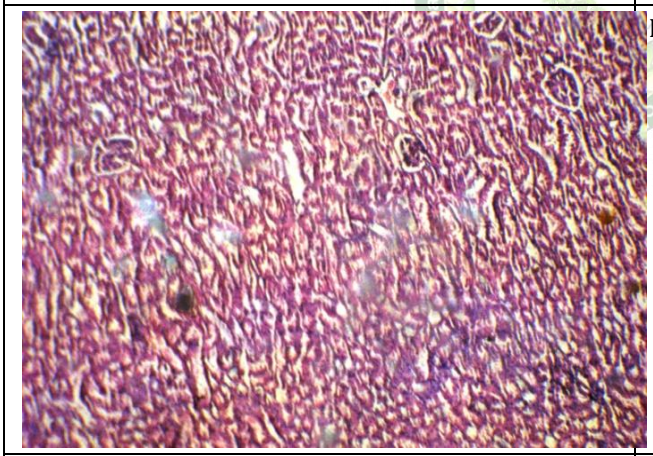

Fig 6 : LQ14 – 5 ml/kg induced Kidney

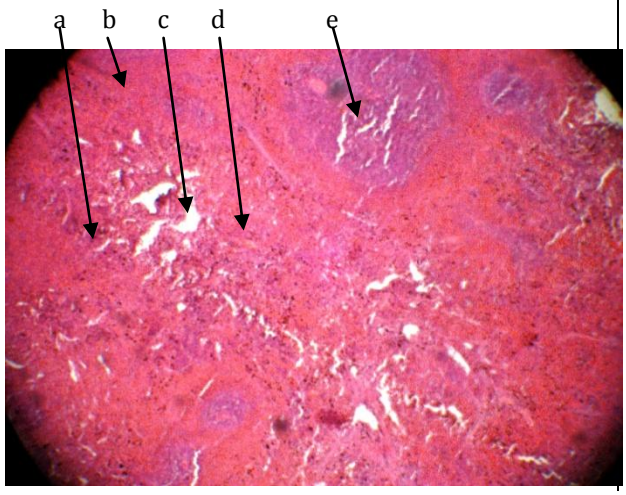

Fig. 8: Normal spleen
HE Stain

a. **Trabuculae**
b. **Capsule**
c. **Central arteriole**
d. **White pulp**
e. **Red pulp**

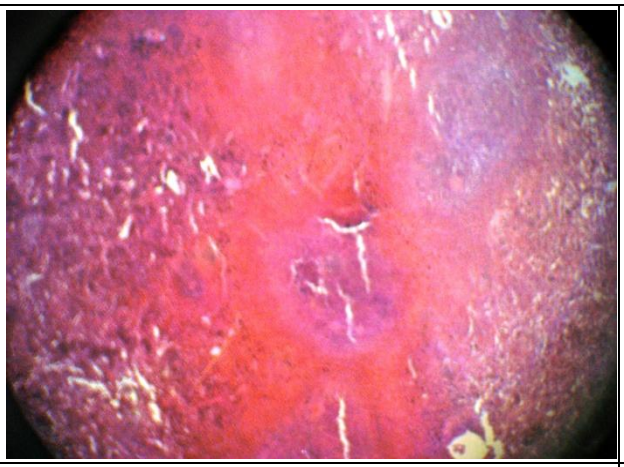

Fig. 9: LQ14 – 2.5 ml/kg induced Spleen

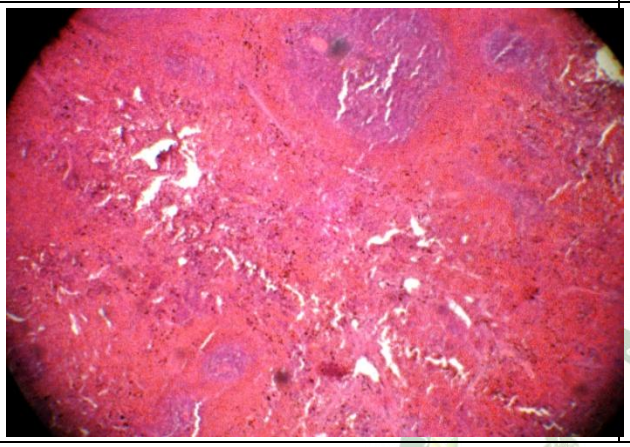

Fig. 10: LQ14 – 5 ml/kg induced Spleen

DISCUSSION

The polyherbal test formulation LQ 14 has been prepared with the medicinal herbs like *Holarrhena antidysenterica, Mangifera indica, Cyperus rotundus, Centella asiatica, Curcuma longa* etc. All these components are well reported for anti-diarrhoeal and anti-microbial activities and used in the Indian subcontinent. [13-15] But, in combination (LQ14), it has not been used earlier. The test formulation in this study did not reveal any signs of toxicity or mortality up to 10 ml/kg per oral dose in mice and rats. The percentages of dead were corrected and the probit values were also determined but LD 50 could not be determined. The dose up to 10 ml/kg orally (extract) in mice and rat is safe and practically non-toxic. Further, behavioral observations related to sign of toxicity (activity, tremors, writhing, sedation, ptosis, lacrimation, diarrhoea etc.) were not noted in any animal of both the species up to 10 ml/kg per oral dose. In this study, 4 weeks repeated oral dose of LQ14 obtained no evidence of adverse health effects in rats. During the course of investigation, no significant biological differences were observed in terms of body weight, food and water consumption and its efficiency among tested rats. In addition, clinical observations, behavioral assessment, and organ weights were not changed in all LQ14 treatment groups. No significant biological differences in hematological profiles, biochemical parameters and histological microscopic findings were observed. The absence of biologically significant differences in these toxicological endpoints supports the safety of LQ14 usage. All these findings collectively indicate that does not exert toxic effects in rats up to a concentration of 5 ml/kg/day upon 4 weeks repeated oral administration.

CONCLUSION

The herbal test product, LQ14 could be assumed to have no lethal effects and is safe for oral use. The absence of biologically significant differences in these toxicological endpoints supports the safety of LQ14 usage in patients of acute diarrhoea.

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Authors' contributions

SM and MN were responsible for the acquistion and analysis of the experimental data, RSD was responsible for coordination and writing of the manuscript. TS did the histopathological evaluation. SB assisted in the histopathological evaluation and revision of the manuscript. SM, MN, SB and RSD participated in the design of the study and performed the critical revision of the manuscript. All authors read and approved the final manuscript

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